Immune modulation by *Bacillus subtilis*-based direct-fed microbials in commercial broiler chickens$^{\diamond}$

Kyung-Woo Lee [a,b,1], Duk Kyung Kim [a,c,1], Hyun S. Lillehoj [a,*], Seung I. Jang [a,2], Sung-Hyen Lee [a,d]

[a] *Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, USDA, Beltsville, MD 20705, USA*
[b] *Department of Animal Science and Technology, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Republic of Korea*
[c] *Animal Genomics and Breeding Center, Hankyong National University, Anseong 456-749, Republic of Korea*
[d] *National Academy of Agricultural Science, Rural Development Administration, Wangju-gun, Jeollabuk-do 565-851, Republic of Korea*

ARTICLE INFO

ABSTRACT

Direct-fed microbials (DFMs), also known as probiotics, have been successfully used to improve the balance of gut microbiota. Spores of *Bacillus subtilis*, have been used as DFMs for food animals and humans and our previous studies showed that dietary supplementation of broiler chickens with a *B. subtilis* DFM from hatch enhanced innate immunity and improved protection against avian coccidiosis. In two experiments, we investigated the effects of dietary *B. subtilis*-based DFM on growth performance and host humoral immune response to *Eimeria* spp. and *Clostridium perfringens*, and evaluated *B. subtilis*-induced global gene expression changes in broiler chickens. Day-old broiler chickens were fed diets with or without DFMs for 28 days starting from hatch. At 14 and 28 days post-hatch, broiler chickens fed the DFM-enriched diet gained significantly more weight (P<0.05) compared with the control diet-fed chickens. Broiler chickens fed diet containing DFM exhibited a significant decrease (P=0.033) in *Eimeria*- and *C. perfringens* necrotic enteritis B-like (NetB) toxin-specific serum antibodies compared with those fed a control diet at day 28. The levels of transcripts encoding interleukin (IL) 1β, IL12 and interferon-γ were greater (P=0.004) in the DFM-fed chickens compared with the control chickens. In order to better understand *B. subtilis*-induced molecular changes associated with improved innate immunity, mRNA was isolated from mid-intestine of DFM-fed chickens for global microarray analysis. Dietary DFMs induced considerable changes in transcriptional expression in intestine, and biofunctional analysis identified 37 genes related with "Inflammatory Response". These results indicate that dietary *B. subtilis* DFM is an effective growth-promoting dietary supplement to increase growth and augment innate immune response in broiler chickens. Further study is needed to investigate the mechanism mediated by dietary DFMs to enhance protective innate immunity in a disease model (i.e., necrotic enteritis) which causes considerable economic impact in the poultry industry worldwide.

Published by Elsevier B.V.

$^{\diamond}$ Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

* Corresponding author. Tel.: +1 301 504 8771; fax +1 301 504 5103.
E-mail address: Hyun.Lillehoj@ARS.USDA.GOV (H.S. Lillehoj).

[1] These authors contributed equally to this work.

[2] Current address: Institute of Health and Environment, Daejeon Metropolitan City, Daejeon 305-338, Republic of Korea.

1. Introduction

In Europe and South Korea, growth promoting antibiotics have been banned since 2006 and 2012, respectively, and such bans are further expected to affect the rest of the world. The development of alternatives to antibiotics is becoming the mainstream topic for animal science research involved in developing sustainable animal production systems. Similarly, increasing regulations and bans on the use of anticoccidial drugs coupled with financial challenges in developing new drugs and live vaccines increase the need for antibiotic alternative strategies for coccidiosis control (Lillehoj and Lee, 2012; Shirley and Lillehoj, 2012).

In the absence of alternatives to antibiotics, chickens raised under current intensive production systems face a higher risk of infection by enteric pathogens. In the United States, clostridial diseases such as necrotic enteritis (NE) and gangrenous dermatitis (GD), and coccidiosis are among the most important enteric diseases of economic importance in chickens (Lee et al., 2011a). Avian coccidiosis is a well-known parasitic disease of industry concern worldwide and in the United States alone, the cost of anticoccidial medication has been assumed to be US $127 million annually (Chapman, 2009) based on a previous estimation in the UK (Williams, 1999). Avian coccidiosis is caused by at least seven distinct species of *Eimeria* apicomplexan sporozoan that infect various regions of the chicken intestinal mucosa. In addition, pre-exposure to certain species of *Eimeria* has been strongly implicated in promoting NE (Williams et al., 2003) and GD (Li et al., 2010a,b). NE in chickens is caused by *Clostridium perfringens*, a Gram-positive, anaerobic, spore-forming bacterium. Globally, the economic loss attributable to NE in poultry is estimated to cost the US $2 billion annually, largely because of medical treatments and impaired growth performance (Cooper et al., 2009; Van Immerseel et al., 2009; Lee et al., 2011a). GD is caused by *C. perfringens* and *C. septicum*, resulting in red to black areas of moist, gangrenous skin over the breast, abdomen, wing tips, or thighs with feather loss and sloughing of the epidermis. NE generally occurs at 17–18 days of age whereas GD develops later at 4–7 weeks of age. Combined with *C. perfringens*, co-infection with immunosuppressive viruses, re-use of litter, high stocking density, and high protein or high viscosity diets are other common risks factors that have been proposed to contribute to both diseases (Li et al., 2010a,b; Lee et al., 2011a).

Direct-fed microbials (DFMs) are live microorganisms that confer a health benefit on the host by balancing intestinal microbes (FAO/WHO, 2002). Although the concept of DFMs is not new, DFMs have been used as prophylactic agents against enteric pathogens, presumably either by balancing microbiota, or by modulating host immunity, or both (Callaway et al., 2008; Flint and Garner, 2009; Applegate et al., 2010; Lee et al., 2010c). In our laboratory, dietary DFMs have been investigated as an alternative strategy to reduce the effect of avian coccidiosis (Lee et al., 2010a,b; Lillehoj and Lee, 2012). For example, it has been shown that dietary *B. subtilis* reduced the clinical signs of experimentally induced avian coccidiosis which correlated with increased innate and acquired immunity in broiler chickens (Lee et al., 2010a,b). In addition to coccidiosis, dietary DFMs reduced clinical signs of enteric diseases such as *Salmonella* spp. or clostridial disease, i.e., NE (Geier et al., 2010; Chen et al., 2012; El Kady et al., 2012; Levkut et al., 2012; Zhang et al., 2012) in chickens. As expected, DFMs modulated host protective immunity against *Salmonella* infection (Higgins et al., 2011; Levkut et al., 2012).

To gain better insight on the role of *B. subtilis*-based DFMs, two separate experiments were conducted to firstly evaluate the effects of dietary DFMs on growth performance, antibody response and innate immunity, and secondly to investigate global gene expression induced by dietary *B. subtilis* in broiler chickens.

2. Materials and methods

2.1. DFMs and experimental diets

A multi-strain *B. subtilis*-based commercial DFM product (Avicorr™, Danisco, Waukesha, WI) was used in this study. *B. subtilis* strains were isolated from various sources including poultry litter or other agricultural environments. They were selected based on their inhibitory effects on avian pathogenic *Escherichia coli* or *C. perfringens* type A (Lee et al., 2010a). This product is generally recognized as safe by the US Food and Drug Administration and is approved for dietary application to animals by the Association of American Feed Control Officials. Basal diet was in a non-medicated mash form consisting of corn, soybean meal, poultry and animal byproduct, and distillers dried grains with solubles. The DFM diet was formulated by mixing the basal diet with DFMs to contain 1.5×10^5 cfu/g of diet as per the manufacturer's recommendation. The control diet was formulated by mixing the base diet with carrier only. All experimental protocols were approved by the Small Animal Care Committee of Beltsville Agricultural Research Center and the University of Delaware.

2.2. Experiment 1: DFM feeding trial

2.2.1. Experimental design

A feeding experiment was conducted at the University of Delaware research facility. Three hundred 1-day-old broiler chickens (Ross 708) were used in this study. The experiment consisted of two dietary treatments and each treatment had six pens as replicate. Upon arrival, they were randomly placed (25 chicks/pen) on the floor pens with used litters and subjected to either DFM-enriched or standard control diet. Used litter was obtained from a commercial broiler farm with endemic GD, homogenized and evenly distributed into pens. The presence of six different species, i.e., *Eimeria acervulina*, *Eimeria brunette*, *Eimeria maxima*, *Eimeria mitis*, *Eimeria praecox*, and *Eimeria tenella* in the used litter were confirmed by PCR-based

Table 1
Oligonucleotide primers used for quantitative RT-PCR.

Symbol	Entrez gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')	GeneBank No.
IL1β[a]	Interleukin 1, beta	TCTGGGACCACTGTATGCTCT	ACACCAGTGGGAAACAGTATCA	NM_204524
IFNγ[a]	Interferon, gamma	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	NM_205149
IL10[a]	Interleukin 10	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	NM_001004414
IL8[a]	Interleukin 8	GGCTTGCTAGGGGAAATGA	AGCTGACTCTGACTAGGAAACTGT	NM_204608
IL6[a]	Interleukin 6	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT	NM_001277996
IL17F[a]	Interleukin 17F	CTCCGATCCCTTATTCTCCTC	AAGCGGTTGTGGTCCTCAT	NM_204460
TNFSF15[a]	Tumor necrosis factor (ligand) superfamily, member 15	CCTGAGTATTCCAGCAACGCA	ATCCACCAGCTTGATGTCACTAAC	NM_001024578
IL2[a]	Interleukin 2	TCTGGGACCACTGTATGCTCT	ACACCAGTGGGAAACAGTATCA	NM_204153
IL12[a]	Interleukin 12	AGACTCCAATGGGCAAATGA	CTCTTCGGCAAATGGACAGT	NM_213571
IL4[a]	Interleukin 4	ACCCAGGGCATCCAGAAG	CAGTGCCGGCAAGAAGTT	NM_001007079
IL13[a]	Interlerkin 13	CCAGGGCATCCAGAAGC	CAGTGCCGGCAAGAAGTT	NM_001007085
ACSL4[b]	Acyl-CoA synthetase long-chain family member 4	GCAAGCATTCAGCCAAGCAG	TGCAGCCGTAAGTGATGCAA	XM_420317.2
ALDH3A2[b]	Aldehyde dehydrogenase 3 family, member A2	GAGAACATCAAGGCGACGCT	TTGACTCCGCAGAAACGTCC	NM_001006223.1
CIDEA[b]	Cell death-inducing DFFA-like effector a	CGGTGCTGCGATCTTTGGTA	CTGTGTCCACAACTGTGCCA	XM_001231679.1
FABP4[b]	Fatty acid binding protein 4, adipocyte	ACCAGGAAGATGGCTGGTGT	TTGCCATCCCACTTCTGCAC	NM_204290.1
GSTT1[b]	Glutathione S-transferase theta 1	ATCCTTACAGGCCAGCCACT	CCAGTACGCTAACTTGGGCC	XM_001231999.1
ITGB6[b]	Integrin, beta 6	TGGACAGTAAGCTGGCAGGA	CTCCTGCAGTCGACCAACTG	XM_422037.2
SCARB1[b]	Scavenger receptor class B, member 1	GGAGCGAAGCCAAAGGTGAA	CCATCATGACAGCTGCTCCC	XM_415106.2
SPI1[b]	Spleen focus forming virus (SFFV) proviral integration oncogene spi1	AGCCCACCACTGGAGGTATC	CAGCGATGTGCCAATGCTTC	NM_205023.1
TRH[b]	Thyrotropin-releasing hormone	ACATGCCTCTGCCACAATGG	GTCCCACAGTGACCTTCTGC	NM_001030383.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA	NM_204305

[a] Primers used for cytokine mRNA in DFM feeding trial.
[b] Primers used for validation of microarray data in DFM global gene expression trial.

species-specific ITS-1 sequences of rDNA as previously described (Lee et al., 2012). No further attempt was taken to demonstrate the presence of *Clostridium* sp. in the litter. The feeding trial lasted 28 days and individual body weights were measured at days 14 and 28.

At 14 and 28 days post-hatch, five birds from each pen (*n* = 6 pens/treatment) were randomly selected and used for blood sampling after cervical dislocation. Immediately after blood sampling, small intestine was excised and used for RNA isolation. Sera were obtained by gentle centrifugation (600 × *g* for 15 min) and stored at −20 °C until use. Total RNA was extracted from the intraepithelial lymphocytes (IELs) from 5-cm segments of mid-jejunum and ileum using TRIzol (Invitrogen) as described (Hong et al., 2006; Park et al., 2008). The IELs were isolated by density gradient centrifugation as described by Lee et al. (2010a).

2.2.2. Serum antibody determination against Eimeria and C. perfringens

Serum antibodies against *Eimeria* spp. and *C. perfringens* were measured by in-house ELISA using recombinant coccidia profilin protein (Lee et al., 2010b) and *C. perfringens* necrotic enteritis B(NetB)-like toxin (Lee et al., 2011c) which were expressed in *E. coli*. A value of optical density at 450 nm was determined with a microplate reader (Bio-Rad Richmond, CA).

2.2.3. Cytokine mRNA assay

Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 μl of 10× reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 μl of stop solution added, and the mixture heated at 70 °C for 10 min. RNA was reverse transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Quantitative RT-PCR (qRT-PCR) oligonucleotide primers for chicken cytokines, chemokines, and GAPDH are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from intestine using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene) as described (Hong et al., 2006; Park et al., 2008). Standard curves were generated using $\log_{10}$ diluted standard RNA and the levels of individual transcripts were normalized to those of GAPDH using the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) values for the amplification products was calculated by pooling values from all samples in that experiment.

2.2.4. Statistical analysis

Pen was considered as the experimental replicate. The data were evaluated by one-way ANOVA using the program SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). The cytokine expression data to be compared were subjected to normality test (Kolmogorov–Smirnov test). When the data were normally distributed, statistical comparisons were performed using the Student t-test. When the data deviated from normality, a non-parametric, Mann–Whitney t-test was used for comparisons. A value of P<0.05 was considered significant.

2.3. Experiment 2: effect of dietary B. subtilis on genome-wide gene expression in the intestine

2.3.1. Experimental design

Fifty one-day-old Ross broiler chickens (Longenecker's Hatchery, Elizabethtown, PA) were randomly housed in 10 Peter-sime brooder units (five chicks per pen) and subjected to either DFM or control diet *ad libitum*. Water was available *ad libitum*. The feeding trial lasted 21 days, of which duration would exhibit the DFM effect on global gene expression in the intestine. At day 21, total RNA was extracted from the IELs of three birds per pen (three chicks per pen, five pens per treatment) from 5-cm segments of mid-jejunum and ileum using TRIzol (Invitrogen) as described earlier in Section 2.2.1 and pooled into each treatment groups with equal amount of RNA ($n = 5$). The IELs were isolated by density gradient centrifugation as described (Lee et al., 2010a). The concentration and the integrity of RNA were determined using Agilent bioanalyzer and RNA integrity number (RIN) software (RIN > 7.0). Global gene expression analysis was carried out as described by Kim et al. (2011). In brief, RNAs isolated were amplified using the Two-Color Quick Amp Labeling Kit (Agilent Technologies) with cyanine 3 (Cy3)- or Cy5-labeled CTP. The probes from the control (basal diet) and treatment (DFM diet) groups labeled with the two different colors were hybridized with a Chicken Gene Expression Microarray (Agilent Technologies, Santa Clara, CA) containing 43,803 elements. The replicate was conducted with alternation of Cy3- and Cy5-labeled RNAs to prevent data distortion from sample labeling (McShane et al., 2003). Microarray images were scanned, and data extraction and analysis were performed using Feature Extraction software version 10.7.3.1 (Agilent Technologies).

2.3.2. Microarray data analysis and validation by qRT-PCR

GeneSpring GX10 software (Silicon Genetics) was used to normalize image analysis data and to determine the fold changes in gene expression as previously described (Kim et al., 2012). Flag information, the feature quality data from the signals on microarray, was applied to strain the spots with 100% valid values from each sample and an asymptotic t-test analysis was performed to analyze the significance between the non-supplemented diet and DFM-supplemented diet groups. To generate signal ratios, signal channel values from DFM-fed birds were divided by values from negative control. Modulated elements were defined as RNAs with ≥2.0-fold increased or decreased levels with P<0.05 as determined by the Student's t-test. All microarray information and data were deposited in the Gene Expression Omnibus database (series record number, GSE41934). The differentially expressed genes between the control and DFM-fed groups were analyzed using Ingenuity Pathway Analysis software (http://www.ingenuity.com) where each identifier was mapped to its corresponding gene. Both 2.0-fold increased and 2.0-fold decreased identifiers were defined as value parameters for the analysis. Functional gene analysis was performed to identify the biological functions of genes from the mapped datasets. Microarray data were further validated by qRT-PCR as described in Section 2.2.3 and the oligonucleotide primers for selected transcripts are listed in Table 1.

3. Results

3.1. Effect of dietary DFMs on growth performance

At 14 and 28 days post-hatch, broiler chickens fed the *B. subtilis*-supplemented diet were heavier (P<0.05) compared with the control diet-fed chickens (Table 2).

3.2. Effect of dietary DFMs on serum antibody levels against Eimeria and C. perfringens NetB toxin

Serum profilin-reactive antibodies were low at day 14 and significantly (P<0.05) increased at day 28 (Table 2). There was no statistical difference (P=0.319) in profilin-specific antibody levels between dietary treatments at day 14. However, at day 28, broiler chickens fed DFM-supplemented diet exhibited lower (P=0.033) profilin-specific serum antibodies compared with those fed a control diet. At day 28, antibodies against *C. perfringens* NetB toxin were lower (P=0.028) in chickens fed DFM-containing diets compared with the control diet-fed chickens (Table 2). No difference in *C. perfringens* NetB toxin was noted at day 14.

3.3. Effect of dietary DFMs on cytokine transcript levels in intestinal IELs

To gain better insight on the role of *B. subtilis*-based DFMs on immune function, the level of various cytokines was quantified by real time RT-PCR at day 28. Of the cytokines selected, intestinal IEL IFNγ, IL1β and IL12 transcript levels were greater (P<0.05) in *B. subtilis*-fed chickens compared with the control chickens (Table 3). On the other hand, no significant

Table 2

Effect of dietary *B. subtilis* on body weight and serum antibody levels against profilin and *Clostridium perfringens* necrotic enteritis B-like toxin in broiler chickens (experiment 1).

	Control		DFM		P value
	Mean	SD	Mean	SD	
Body weight, g/bird					
Day 14	352.6[a]	5.07	366.6	9.82	0.012
Day 28	1196.4	43.13	1264.9	38.03	0.033
Profilin-specific antibody levels[b]					
Day 14	0.45[a]	0.043	0.48	0.053	0.319
Day 28	1.20	0.355	0.80	0.126	0.033
Clostridium perfringens necrotic enteritis B-like toxin-specific antibody levels[b]					
Day 14	0.55[a]	0.115	0.52	0.066	0.573
Day 28	0.82	0.043	0.68	0.095	0.028

[a] Values are means with standard deviation (SD) of six replicates ($n = 6$) per treatment.

[b] ELISA optical density at 450 nm (OD_{450}) values corrected for background reactivity.

Table 3

Effect of dietary *B. subtilis* on expression levels of cytokine mRNA transcripts in intraepithelial lymphocytes in broiler chickens (experiment 1).

Cytokine	Control		DFM		P value
	Mean	SD	Mean	SD	
IFNγ[c]	4.20E−05[a,b]	2.44E−05	1.13E−04	4.93E−05	0.041
IL1β[d]	1.35E−04	2.88E−05	2.62E−04	5.80E−05	0.010
IL12[c]	1.35E−11	2.68E−12	4.31E−11	4.77E−12	0.001

[a] Values are means with standard deviation (SD) of six replicates ($n = 6$) per treatment.

[b] Values are expressed as gene/GAPDH which is the mean expression ratio that normalized the values of target gene by GAPDH (reference gene) expression value.

[c] Student *t*-test.

[d] Mann–Whitney *t*-test.

effect of dietary *B. subtilis* on the expression levels of cytokines encoding for IL2, IL4, IL6, IL8, IL10, IL13, IL17F and TNFSF15 was observed (data not shown).

3.4. Effects of dietary DFMs on global gene expression

When comparing *B. subtilis*-fed chickens vs. controls, 906 microarray elements were significantly altered with >2.0-fold changes (P<0.05) with −43.70- to 6.39-fold changes. Of these, 497 mRNAs were increased and 409 were decreased. This dataset was mapped to the human, mouse, rat and chicken genomes using Ingenuity Knowledge Base software, leading to the identification and annotation of 339 chicken genes (131 increased and 208 decreased). Among annotated genes, 10 most up- or down-regulated genes are listed in Table 4.

To determine the biologically relevant characteristics of these differentially expressed genes, biological function analysis was performed using the Ingenuity Knowledge Base. Biofunctional analysis identified 37 genes related to inflammatory response which were grouped into this function are listed in Table 5.

3.5. Validation of gene expression microarray data by qRT-PCR

The expression patterns observed by microarray analysis were validated by qRT-PCR with five selected transcripts (Table 1) from the pathway of "LPS/IL-1 Mediated Inhibition of RXR Function" and four immune-related genes, whose levels were significantly modulated when comparing *B. subtilis*-fed vs. control chickens. These genes were acyl-CoA synthetase long-chain family member 4 (ACSL4), aldehyde dehydrogenase 3 family, member A2 (ALDH3A2), cell death-inducing DFFA-like effector a (CIDEA), fatty acid binding protein 4 (FABP4), glutathione S-transferase theta 1 (GSTT1), integrin, beta 6 (ITGB6), scavenger receptor class B, member 1 (SCARB1), spleen focus forming virus (SFFV) proviral integration oncogene spi1 (SPI1) and thyrotropin-releasing hormone (TRH). The expression levels of all nine transcripts that were up-or down-regulated by gene expression analysis were validated and confirmed by qRT-PCR (Fig. 1). As previously discussed, the differences in the magnitude of the changes observed by the two techniques might be due to differences in the normalization methods used and/or the different fluorescent dyes used (Lee et al., 2002).

4. Discussion

Our results indicate that dietary *B. subtilis* when used in a broilers diet as a potential growth promoter at hatch, can provide beneficial effects on broiler performance and modulate host humoral and cellular immune responses to enteric pathogens.

Table 4
The most up- and down-regulated genes induced by dietary *B. subtilis* in chickens (experiment 2).

| Symbol	Gene Name	Fold Change	Location	Type(s)
Up-regulated				
PNLIP	Pancreatic lipase	84.13	Extracellular space	Enzyme
CELA2A	Chymotrypsin-like elastase family, member 2a	64.603	Extracellular space	Peptidase
CPA1	Carboxypeptidase a1 (pancreatic)	56.997	Extracellular space	Peptidase
CTRC	Chymotrypsin C (caldecrin)	55.321	Extracellular space	Peptidase
CLPS	Colipase, pancreatic	40.684	Extracellular space	Other
CEL	Carboxyl ester lipase (bile salt-stimulated lipase)	35.953	Extracellular space	Enzyme
PDIA2	Protein disulfide isomerase family A, member 2	34.781	Cytoplasm	Enzyme
A2ML1	Alpha-2-macroglobulin-like 1	18.804	Cytoplasm	Other
MMS22L	MMS22-like, DNA repair protein	18.743	Nucleus	Other
HBE1	Hemoglobin, epsilon 1	17.402	Cytoplasm	Transporter
CDH3	Cadherin 3, type 1, P-cadherin (placental)	16.036	Plasma membrane	Other
AMY2A	Amylase, alpha 2A (pancreatic)	15.74	Extracellular space	Enzyme
CSTB	Cystatin B (stefin B)	13.6	Cytoplasm	Other
INS	Insulin	13.024	Extracellular space	Other
EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1	12.979	Cytoplasm	Translation regulator
Down-regulated				
RIOK3	RIO kinase 3 (yeast)	−11.364	Unknown	Kinase
NMNAT1	Nicotinamide nucleotide adenylyltransferase 1	−10.898	Nucleus	Enzyme
HSPA2	Heat shock 70 kDa protein 2	−9.184	Cytoplasm	Other
RGCC	Regulator of cell cycle	−8.525	Cytoplasm	Other
FABP4	Fatty acid binding protein 4, adipocyte	−6.176	Cytoplasm	Transporter
PMP22	Peripheral myelin protein 22	−6.131	Plasma membrane	Other
KRT23	Keratin 23 (histone deacetylase inducible)	−5.985	unknown	Other
FGL1	Fibrinogen-like 1	−5.719	Extracellular space	Other
GPRC5A	G protein-coupled receptor, family C, group 5, member A	−5.593	Plasma Membrane	G-protein coupled receptor
PDK4	Pyruvate dehydrogenase kinase, isozyme 4	−5.548	Cytoplasm	Kinase
SEMA6D	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6D	−5.534	Plasma membrane	Other
CHRDL1	Chordin-like 1	−5.301	Extracellular space	Other
RAB30	RAB30, member RAS oncogene family	−5.196	Cytoplasm	Enzyme
CLEC3B	C-type lectin domain family 3, member B	−4.932	Extracellular space	Other
DDX4	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	−4.837	Nucleus	Enzyme
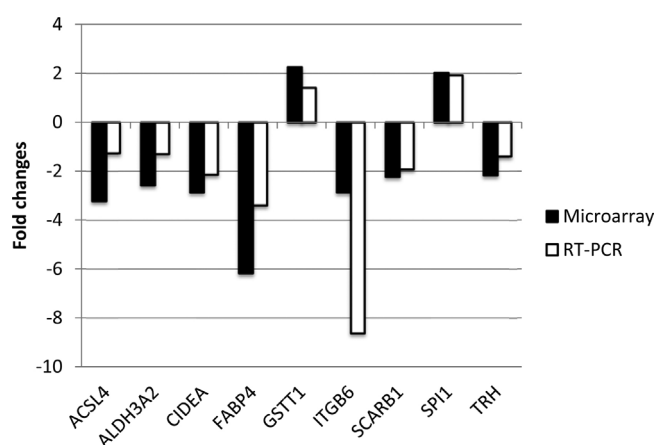
Fig. 1. Comparison between microarray analysis and qRT-PCR for levels of mRNAs corresponding to selected genes (experiment 2). Each bar represents the ratio of mRNA levels in the chickens fed *B. subtilis* vs. non-treated controls. Acyl-CoA synthetase long-chain family member 4 (ACSL4), aldehyde dehydrogenase 3 family, member A2 (ALDH3A2), cell death-inducing DFFA-like effector a (CIDEA), fatty acid binding protein 4, adipocyte (FABP4), glutathione S-transferase theta 1 (GSTT1), integrin, beta 6 (ITGB6), scavenger receptor class B, member 1 (SCARB1), spleen focus forming virus (SFFV) proviral integration oncogene spi1 (SPI1), and thyrotropin-releasing hormone (TRH).

Table 5

The differentially expressed genes which were annotated as the function of "Inflammatory Response" in chickens fed *B. subtilis* compared with a non-supplemented diet (experiment 2).

Symbol	Entrez gene name	Fold change	Location	Type(s)
ALB	Albumin	10.358	Extracellular space	Transporter
CELA1	Chymotrypsin-like elastase family, member 1	8.334	Extracellular space	Peptidase
APCS	Amyloid P component, serum	5.127	Extracellular space	Other
NOS2	Nitric oxide synthase 2, inducible	4.838	Cytoplasm	Enzyme
CSF3	Colony stimulating factor 3 (granulocyte)	4.526	Extracellular space	Cytokine
IL11RA	Interleukin 11 receptor, alpha	4.461	Plasma membrane	Transmembrane receptor
CCL20	Chemokine (C–C motif) ligand 20	4.398	Extracellular space	Cytokine
E2F1	E2F transcription factor 1	4.08	Nucleus	Transcription regulator
SELP	Selectin P (granule membrane protein 140 kDa, antigen CD62)	3.816	Plasma membrane	Other
PLCG1	Phospholipase C, gamma 1	3.372	Cytoplasm	Enzyme
S100A9	S100 calcium binding protein A9	3.184	Cytoplasm	Other
SCARB1	Scavenger receptor class B, member 1	2.942	Plasma membrane	Transporter
MAN2A1	Mannosidase, alpha, class 2A, member 1	2.749	Cytoplasm	Enzyme
CD4	CD4 molecule	2.691	Plasma membrane	Transmembrane receptor
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	2.302	Extracellular space	Cytokine
SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1	2.031	Nucleus	Transcription regulator
TFF2	Trefoil factor 2	2.031	Extracellular space	Other
NTS	Neurotensin	−2.114	Extracellular space	Other
PRDX6	Peroxiredoxin 6	−2.125	Cytoplasm	Enzyme
VEGFA	Vascular endothelial growth factor A	−2.15	Extracellular space	Growth factor
WNT5A	Wingless-type MMTV integration site family, member 5A	−2.289	Extracellular space	Cytokine
CCR8	Chemokine (C–C motif) receptor 8	−2.328	Plasma membrane	G-protein coupled receptor
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	−2.487	Nucleus	Transcription regulator
NR2C2	Nuclear receptor subfamily 2, group C, member 2	−2.537	Nucleus	Ligand-dependent nuclear receptor
ABR	Active BCR-related	−2.608	Cytoplasm	Other
DCN	Decorin	−2.642	Extracellular space	Other
NFIL3	Nuclear factor, interleukin 3 regulated	−2.66	Nucleus	Transcription regulator
HNF4A	Hepatocyte nuclear factor 4, alpha	−2.675	Nucleus	Transcription regulator
CALB1	Calbindin 1, 28 kDa	−2.693	Cytoplasm	Other
PTK2B	PTK2B protein tyrosine kinase 2 beta	−2.743	Cytoplasm	Kinase
NDRG1	N-myc downstream regulated 1	−2.764	Nucleus	Kinase
NFRKB	Nuclear factor related to kappaB binding protein	−2.828	Nucleus	transcription regulator
ITGB6	Integrin, beta 6	−2.874	Plasma membrane	Other
GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	−3.261	Cytoplasm	Peptidase
EPHA4	EPH receptor A4	−4.528	Plasma membrane	Kinase
RGS1	Regulator of G-protein signaling 1	−4.732	Plasma membrane	Other
FABP4	Fatty acid binding protein 4, adipocyte	−6.176	Cytoplasm	Transporter

Previously, we investigated the effects of dietary DFMs on growth promotion and immune modulation and disease resistance to coccidiosis in broiler chickens raised on used litter (Lee et al., 2012). In addition, we found that GD-litter raised broiler chickens produced elevated serum antibody titers against *Eimeria* profilin antigen, but not against antigens of *Clostridium* spp. when compared with those raised on fresh litter, indicating the consistent exposure to the pathogens present in used litter (Lee et al., 2011b). In our study, dietary *B. subtilis* significantly lowered serum antibodies against *Eimeria* profilin and *C. perfringens* NetB toxin compared with the control diet-fed chickens. Thus, one of the plausible explanations on the growth promoting effect induced by dietary DFMs may be related to its effect on enteric pathogens such as *Eimeria* spp. or pathogenic *Clostridium* spp. present in used litter and/or on local inflammatory response induced by *Eimeria* profilin and *Clostridium* toxins. The latter statement however awaits verification as we did not count the pathogen loads present in the intestine in this study. In addition, a positive DFM effect on growth performance would be seen in the less optimal conditions reared under field conditions or exposed to common enteric infections, or both. Indeed, we demonstrated that dietary *B. subtilis* did not have effect on growth of cage-raised, naïve chickens (Lee et al., 2010a), but counteracted the *Eimeria*-induced growth depression in broiler chickens (Lee et al., 2010b). In parallel with our earlier observation, this current study confirms that dietary DFMs can benefit broiler chickens raised under less optimal conditions, facilitating their growth.

Cytokines are immunoregulatory peptides with relatively small molecular weights that participate in innate and adaptive host immune responses. In this study, among 11 cytokines tested, proinflammatory or T helper type 1 (Th-1) cytokines, i.e., IL1β, IL12 and IFNγ were significantly up-regulated by dietary *B. subtilis* in broiler chickens. IFNγ is a common marker of

cellular immunity where higher levels have been associated with enhanced protective immune responses to coccidiosis (Lillehoj and Choi, 1998). IL1β is a major proinflammatory cytokine that mediates innate immunity and is produced by macrophages, monocytes and dendritic cells. IL12 is an important cytokine required for the initiation and regulation of cellular immunity through the differentiation of naïve T cells into Th-1 cells, which is crucial for host resistance to many microbial pathogens (Park et al., 2008). Recently, Rajput et al. (2013) have reported that *Bacillus*-based DFM significantly induced inflammatory and anti-inflammatory cytokines in jejunum and ileum of broiler chickens. Thus, our result shows that dietary *B. subtilis* immunologically modulated the host to increase its defense capability against coccidiosis and *C. perfringens*-associated infection. This conclusion is supported by the increased body weights and enhanced gene expression of major innate immunity genes which are involved in initiating and regulating immune response against *Eimeria* and *C. perfringens* in *B. subtilis*-fed chickens compared with the control chickens.

The current findings confirmed the previous studies that dietary *B. subtilis* could modulate immune response in broiler chickens (Lee et al., 2010a,b,c). Thus, we further investigated to see the genome-wide transcriptional changes offered by dietary *B. subtilis* in broiler chickens using 43K chicken gene expression microarray. Due to the lack of the number of genes annotated to the gene name and function, the differentially expressed transcripts were mapped to human, mouse and rats as well as chicken genome to enrich the data for biological function analysis. The biological function analysis revealed that dietary *B. subtilus* treatment most significantly altered the genes having the function of "Inflammatory Response" in the category of "Disease and Disorders". Among the genes related with this function (Table 5), inducible nitric oxide synthase 2 (iNOS) produces nitric oxide (NO) and is rapidly induced upon exposure to a variety of inflammatory agents including allergens, oxidants or cytokines (Naura et al., 2010). In a previous study, we have shown that NO increased following dietary *B. subtilis* treatment in chicken IEL (Lee et al., 2010a). NO inductions by other DFMs, *Bifidobacterium* and *Lactobacillus*, were also reported elsewhere (Korhonen et al., 2001; Kim et al., 2007). NO has been recognized to play the important roles in the immune response such as pathogenesis, control of infectious diseases, tumors, and autoimmune processes (Bogdan, 2001), therefore dietary *B. subtilis* might induce iNOS expression following NO production in chicken gut mucosa. Tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15) was reported as a pro-inflammatory cytokine and plays an important role in the local inflammatory response to *Eimeria* in chickens (Park et al., 2007; Takimoto et al., 2008). TNFSF15 stimulated T cell proliferation in response to IL-2, and induces secretion of IFNγ and granulocyte-macrophage colony stimulating factor and also synergized with IL-12/IL-18 to augment IFNγ production in human peripheral blood T cells and NK cells (Zhang and Li, 2012). In this study, dietary *B. subtilis* induced the up-regulation of TNFSF15 in chicken IELs as in our previous report (Lee et al., 2010a) and the increase of IFNγ was validated by qRT-PCR. Additionally, genes with function of "Inflammatory Response" such as FABP4 and SCARB1 were downregulated. Further studies are needed to investigate the relationship between inflammatory responses with growth traits which is beyond the scope of the current studies. Systematic studies using immunology (i.e., gene expression profile) and nutrition (i.e., net energy measurement) tools would provide more clues on this matter.

Of interest, among the most up-regulated genes by DFM were those related to feed digestion such as pancreatic lipase (PNLIP), carboxypeptidase (CPA1), chymotrypsin-like elastase family (CELA2A), chymotrypsin (CTRC), lipase (CEL), colipase (CLPS) and amylase (AMY2A) in the gut. DFM-induced increase in digestive enzyme activity in broiler chickens has been reported (Wang and Gu, 2010). To our knowledge, this is the first report to show the molecular basis that DFM could stimulate the production of digestive enzymes by upregulating the genes encoding the nutrients-digesting enzymes in the intestine.

5. Conclusions

The results of this study indicated that dietary *B. subtilis* administered to newly hatched chickens resulted in the considerable changes of transcriptional expression in chicken IELs. In addition, continuous feeding of dietary *B. subtilis* from hatch significantly increased body weight gain and enhanced innate immunity upon infection with *Eimeria* spp. or *Clostridium* spp. when grown on used litter. Finally, proinflammatory or Th-1, but not Th-2 or chemokine transcripts were significantly up-regulated by dietary *B. subtilis*. Collectively, our study provides the evidence that *B. subtilis*-based DFMs possess immune-modulating properties and growth enhancing capacity in broiler chickens.

Conflict of interest

The authors are not aware of any conflicts of interest associated with this manuscript.

Acknowledgments

in Lasher Poultry Diagnostic Laboratory, University of Delaware, and Dr. Gregory R. Siragusa in Danisco for providing us the experimental facility and the experimental diets for this study.

References

Applegate, T.J., Klose, V., Steiner, T., Ganner, A., Schatzmayr, G., 2010. Probiotics and phytogenics for poultry: Myth or reality? J. Appl. Poult. Res. 19, 194–210.

Bogdan, C., 2001. Nitric oxide and the immune response. Nat. Immunol. 2, 907–916.

Callaway, T.R., Edrington, T.S., Anderson, R.C., Harvey, R.B., Genovese, K.J., Kennedy, C.N., Venn, D.W., Nisbet, D.J., 2008. Probiotics: prebiotics and competitive exclusion for prophylaxis against bacterial disease. Anim. Health Res. Rev. 9, 217–225.

Chapman, H.D., 2009. A landmark contribution to poultry science-prophylactic control of coccidiosis in poultry. Poult. Sci. 88, 813–815.

Chen, C.Y., Tsen, H.Y., Lin, C.L., Yu, B., Chen, C.S., 2012. Oral administration of a combination of select lactic acid bacteria strains to reduce the Salmonella invasion and inflammation of broiler chicks. Poult. Sci. 91, 2139–2147.

Cooper, K.K., Trinh, H.T., Songer, J.G., 2009. Immunization with recombinant alpha toxin protects broiler chicks against experimental challenge with Clostridium perfringens. Vet. Microbiol. 133, 92–97.

El Kady, M.F., Hassan, E.R., Radwan, I.A.E., Rabie, N.S., Rady, M.M., 2012. Effect of probiotic on necrotic enteritis in chickens with the presence of immun-suppressive factors. Global Vet. 9, 345–351.

FAO/WHO, 2002. Guidelines for the evaluation of probiotics in food. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. Ontario, Canada. Available from: <ftp://ftp.fao.org/es/esn/food/wgreport2.pdf> (accessed 25.03.13).

Flint, J.F., Garner, M.R., 2009. Feeding beneficial bacteria: A natural solution for increasing efficiency and decreasing pathogens in animal agriculture. J. Appl. Poult. Res. 18, 367–378.

Geier, M.S., Mikkelsen, L.L., Torok, V.A., Allison, G.E., Olnood, C.G., Boulianne, M., Hughes, R.J., Choct, M., 2010. Comparison of alternatives to in-feed antimicrobials for the prevention of clinical necrotic enteritis. J. Appl. Microbiol. 109, 1329–1338.

Higgins, S.E., Wolfenden, A.D., Tellez, G., Hargis, B.M., Porter, T.E., 2011. Transcriptional profiling of cecal gene expression in probiotic- and Salmonella-challenged neonatal chicks. Poult. Sci. 90, 901–913.

Hong, Y.H., Lillehoj, H.S., Lillehoj, E.P., Lee, S.H., 2006. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following Eimeria maxima infection of chickens. Vet. Immunol. Immunopathol 114, 259–272.

Kim, D.W., Cho, S.B., Lee, H.J., Chung, W.T., Kim, K.H., Hwangbo, J., Nam, I.S., Cho, Y.I., Yang, M.P., Chung, I.B., 2007. Comparison of cytokine and nitric oxide induction in murine macrophages between whole cell and enzymatically digested Bifidobacterium sp. obtained from monogastric animals. J. Microbiol. 45, 305–310.

Kim, D.K., Lillehoj, H.S., Lee, S.H., Lillehoj, E.P., Bravo, D., 2012. Improved resistance to Eimeria acervulina infection in chickens due to dietary supplementation with garlic metabolites. Br. J. Nutr 109, 76–88.

Kim, D.K., Lillehoj, H.S., Min, W.G., Kim, C.H., Hong, Y.H., Lillehoj, E.P., 2011. Comparative microarray analysis of intestinal lymphocytes following Eimeria acervulina, E maxima or E. tenella infection. PLoS ONE 6, e27712.

Korhonen, R., Korpela, R., Saxelin, M., Maki, M., Kankaanranta, H., Moilanen, E., 2001. Induction of nitric oxide synthesis by probiotic Lactobacillus rhamnosus GG in J774 macrophages and human T84 intestinal epithelial cells. Inflammation 25, 223–232.

Lee, K.W., Lee, S.H., Lillehoj, H.S., Li, G.X., Jang, S.I., Babu, U.S., Park, M.S., Kim, D.K., Lillehoj, E.P., Neumann, A.P., Rehberger, T.G., Siragusa, G.R., 2010a. Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult. Sci. 89, 203–216.

Lee, K.W., Lillehoj, H.S., Jang, S.I., Li, G., Lee, S.H., Lillehoj, E.P., Siragusa, G.R., 2010b. Effect of Bacillus-based direct-fed microbials on Eimeria maxima infection in broiler chickens. Comp. Immunol. Microbiol. Infect. Dis. 33, e105–e110.

Lee, K.W., Lillehoj, H.S., Siragusa, G.R., 2010c. Direct-fed microbials and their impact on the intestinal microflora and immune system of chickens. J. Poult. Sci. 47, 106–114.

Lee, K.W., Lillehoj, H.S., Jeong, W., Jeoung, H.Y., An, D.J., 2011a. Avian necrotic enteritis: experimental models host immunity. pathogenesis, risk factors, and vaccine development. Poult. Sci. 90, 1380–1390.

Lee, K.W., Lillehoj, H.S., Lee, S.H., Jang, S.I., Ritter, G.D., Bautista, D.A., Lillehoj, E.P., 2011b. Impact of fresh or used litter on the post-hatch immune system of commercial broilers. Avian Dis. 55, 539–544.

Lee, K.W., Lillehoj, H.S., Li, G., Park, M.S., Jang, S.I., Jeong, W., Jeong, H.Y., An, D.J., Lillehoj, E.P., 2011c. Identification and cloning of two immunogenic C. perfringens proteins: elongation factor Tu (EF-Tu) and pyruvate:ferredoxin oxidoreductase (PFO) of Clostridium perfringens. Res. Vet. Sci. 91, e80–86.

Lee, K.W., Lillehoj, H.S., Jang, S.I., Pagès, M., Bautista, D.A., Pope, C.R., Ritter, G.D., Lillehoj, E.P., Neumann, A.P., Siragusa, G.R., 2012. Effects of in ovo vaccination and anticoccidials on the distribution of Eimeria spp. in poultry litter and serum antibody titers against coccidia in broiler chickens raised on the used litters. Res. Vet. Sci. 93, 177–182.

Lee, P.D., Sladek, R., Greenwood, C.M., Hudson, T.J., 2002. Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res. 12, 292–297.

Levkut, M., Revajová, V., Lauková, A., Ševčíková, Z., Spišáková, V., Faixová, Z., Levkutová, M., Strompfová, V., Pistl, J., Levkut, M., 2012. Leukocytic responses and intestinal mucin dynamics of broilers protected with Enterococcus faecium EF55 and challenged with Salmonella Enteritidis. Res. Vet. Sci. 93, 195–201.

Li, G., Lillehoj, H.S., Lee, K.W., Jang, S.I., Marc, P., Gay, C.G., Ritter, G.D., Bautista, D.A., Philips, K., Neumann, A.P., Rehberger, T.G., Siragusa, G.R., 2010a. An outbreak of gangrenous dermatitis in commercial broiler chickens. Avian Pathol. 39, 247–253.

Li, G., Lillehoj, H.S., Lee, K.W., Lee, S.H., Park, M.S., Jang, S.I., Bauchan, G.R., Gay, C.G., Ritter, G.D., Bautista, D.A., Siragusa, G.R., 2010b. Immunopathology and cytokine responses in commercial broiler chickens with gangrenous dermatitis. Avian Pathol. 39, 255–264.

Lillehoj, H.S., Choi, K.D., 1998. Recombinant chicken interferon-γ-mediated inhibition of Eimeria tenella development in vitro and reduction of oocyst production and body weight loss following Eimeria tenella challenge infection. Avian Dis. 42, 307–314.

Lillehoj, H.S., Lee, K.W., 2012. Immune modulation of innate immunity as alternatives-to-antibiotics strategies to mitigate the use of drugs in poultry production. Poult. Sci. 91, 1286–1291.

McShane, L.M., Shih, J.H., Michalowska, A.M., 2003. Statistical issues in the design and analysis of gene expression microarray studies of animal models. J. Mammary Gland Biol. Neoplasia 8, 359–374.

Muller, P.Y., Janovjak, H., Miserez, A.R., Dobbie, Z., 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32, 1372–1379.

Naura, A.S., Zerfaoui, M., Kim, H., Abd Elmageed, Z.Y., Rodriguez, P.C., Hans, C.P., Ju, J., Errami, Y., Park, J., Ochoa, A.C., Boulares, A.H., 2010. Requirement for inducible nitric oxide synthase in chronic allergen exposure-induced pulmonary fibrosis but not inflammation. J. Immunol. 185, 3076–3085.

Park, S.S., Lillehoj, H.S., Allen, P.C., Park, D.W., Fitzcoy, S., Bautista, D.A., Lillehoj, E.P., 2008. Immunopathology and cytokine responses in broiler chickens coinfected with Eimeria maxima and Clostridium perfringens with the use of an animal model of necrotic enteritis. Avian Dis. 52, 14–22.

Park, S.S., Lillehoj, H.S., Hong, Y.H., Lee, S.H., 2007. Functional characterization of tumor necrosis factor superfamily 15 (TNFSF15) induced by lipopolysaccharides and Eimeria infection. Dev. Comp. Immunol. 31, 934–944.

Rajput, I.R., Li, L.Y., Xin, X., Wu, B.B., Juan, Z.L., Cui, Z.W., Yu, D.Y., Li, W.F., 2013. Effect of Saccharomyces boulardii and Bacillus subtilis B10 on intestinal ultrastructure modulation and mucosal immunity development mechanism in broiler chickens. Poult. Sci 92, 956–965.

Shirley, M.W., Lillehoj, H.S., 2012. The long view: a selective review of 40 years of coccidiosis research. Avian Pathol. 41, 111–121.

Takimoto, T., Sato, K., Akiba, Y., Takahashi, K., 2008. Role of chicken TL1A on inflammatory responses and partial characterization of its receptor. J. Immunol. 180, 8327–8332.

Van Immerseel, F., Rood, J.I., Moore, R.J., Titball, R.W., 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol. 17, 32–36.

Wang, Y., Gu, Q., 2010. Effect of probiotic on growth performance and digestive enzyme activity of Arbor Acres broilers. Res. Vet. Sci. 89, 163–167.

Williams, R.B., 1999. A compartmentalized model for the estimation of the cost of coccidiosis to the world's chicken production industry. Int. J. Parasitol. 29, 1209–1229.

Williams, R.B., Marshall, R.N., La Ragione, R.M., Catchpole, J., 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the relationships between necrotic enteritis coccidiosis and anticoccidial vaccination of chickens. Parasitol. Res. 90, 19–26.

Zhang, Z., Li, L.Y., 2012. TNFSF15 modulates neovascularization and inflammation. Cancer Microenviron. 5, 237–247.

Zhang, D., Li, R., Li, J., 2012. *Lactobacillus reuteri* ATCC 55730 and L22 display probiotic potential in vitro and protect against *Salmonella*-induced pullorum disease in a chick model of infection. Res. Vet. Sci. 93, 366–373.